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Comprehensive Investigation of the Presence of JC Virus in AIDS Patients With and Without Progressive Multifocal Leukoencephalopathy

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Progressive multifocal leukoencephalopathy (PML), a viral-induced demyelinating disease, is becoming relatively common, while many diagnostic and pathogenetic aspects remain to be clarified. A study was therefore undertaken in 64 AIDS patients suffering from various neurological disorders, including PML (12 subjects), with the specific objective of searching for JC virus (JCV) DNA by nested PCR (n-PCR) in cerebrospinal fluid (CSF), peripheral blood mononuclear cells (PBMCs), and urine collected from all patients. CSF examination, CD4 and CD8 counts, neurological examinations, and neuroradiological investigations were undertaken. JCV DNA was detected in 92% of CSF specimens in 75% of the PBMCs and urine samples from the PML patients, whereas among the non-PML patients JCV DNA was not detected in any CSF samples, but was found in 10% of PBMCs and in 39% of the urine specimens. BKV and JCV DNA viruria was observed simultaneously in 6% of the AIDS patients without PML. The routine CSF tests including IgG oligoclonal bands, the Link, and Tourtellotte IgG indexes, did not show a typical pattern in PML cases. The data obtained clearly indicate that the detection of JCV DNA in CSF constitutes an efficient marker for PML diagnosis. The simultaneous presence of JCV DNA in the CSF, PBMCs, and urine samples from the PML patients, who did not differ from controls with regard to their immunosuppressive status, suggests that JCV could be carried into the central nervous system (CNS) by infected PBMCs. ***J. Med. Virol. 52:235–242, 1997.***

KEY WORDS: human polyomavirus; PML; cerebrospinal fluid; nested PCR

INTRODUCTION

The human polyomaviruses, BK virus (BKV) and JC virus (JCV), are members of the *Papovaviridae* family and are endemic worldwide [Walker and Frisque, 1986]. BKV and JCV infections are usually acquired during the first two decades of life and both viruses, following primary infection, establish latent infection in the kidneys, and may be reactivated primarily in the course of immunosuppressive states [Chesters et al., 1983].

When reactivated, BKV can induce ureteral stenosis in renal allograft recipients [Coleman et al., 1978; Gardner et al., 1984] and hemorrhagic cystitis in recipients of bone marrow transplants [Apperley et al., 1987; Arthur et al., 1986]. BKV DNA has also been detected in brain tissue from normal subjects and AIDS patients [Elsner and Dorries, 1992; Vago et al., 1996] and in brain and pancreatic tumors [Corallini et al., 1987; Dorries et al., 1987].

JC is a neurotropic virus, and in immunosuppressed patients it can induce progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the central nervous system [Padgett and Walker, 1973;

The results of this study were presented in part at the Annual Meeting of the Laboratory of Tumor Cell Biology, Bethesda, Maryland, August 27–September 2, 1995 (Abstract 310), at the First Congress of the European Federation of Neurological Sciences, Marseilles, France, September 9–14, 1995, and at the 1st European Conference on Experimental AIDS Research, Cannes, France, March 10–13, 1996.

The analyses for this study were performed at the Biology Laboratory of the Don C. Gnocchi Foundation, IRCCS (Research Hospital) of Milan.

Informed consent was obtained from all patients prior to participation in the study. The study was conducted in accordance with guidelines established by the Italian Ministry of Health.

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Shah, 1994]. Once considered a rare disease, PML is now quite common, since the advent of the AIDS epidemic affecting 4–5% of AIDS patients, among whom it is also a frequent cause of death [Berger et al., 1987; Gillespie et al., 1991]. As a result of this increased prevalence of PML cases, many aspects of the disease including its pathology, clinical characteristics, and radiological findings, have become known [Major et al., 1992; Berger and Concha, 1995], but many aspects of the pathogenesis of PML still remain to be clarified.

It has been suggested that the JCV circulating in the human population is an archetypal strain that can establish latency in the kidneys [Yogo et al., 1990; Flaegstad et al., 1991; White et al., 1992]. Under particular conditions such as immunosuppression, the archetypal strain may be reactivated and undergo genomic mutation, which allows JCV to reach the brain, infect oligodendrocytes, and induce PML [White et al., 1992]. It has also been suggested that JCV can spread to the brain by means of peripheral blood mononuclear cells (PBMCs), as JCV DNA has been detected in the PBMCs of AIDS patients [Tornatore et al., 1992].

The more recent detection of JCV DNA in the brains of subjects whose deaths were due to diseases unrelated to PML or AIDS, has suggested that JCV could establish latency in the brains of healthy individuals without inducing PML and that under favorable conditions, JCV can be reactivated and induce PML [White et al., 1992; Ferrante et al., 1995; Vago et al., 1996].

The higher frequency of PML observed as a consequence of the AIDS epidemic called for specific noninvasive and sensitive PML diagnostic techniques. In fact, the clinical characteristics of PML in AIDS patients are non-diagnostic and neuroradiological findings are required to reach a clinical diagnosis of PML, although they alone, are not sufficient. Until recently, the diagnosis of PML was based mainly on the detection of JC virions by immunohistochemical staining, electron microscopy, and viral isolation in cell culture on autoptic or bioptic tissues [Padget and Walker, 1973]. More promising results [Berger and Concha, 1995] have been offered by the more recent development of molecular techniques such as the polymerase chain reaction (PCR) [Saiki et al., 1988] for the detection of JCV in cerebrospinal fluid (CSF).

This report consists of the description of a systematic study of the incidence of JCV and BKV DNA in CSF, PBMCs, and urine specimens from a large group of AIDS patients. The sample population consisted of a group of PML patients (12 subjects) and a group of AIDS patients without PML (52 subjects), but suffering from various other neurological diseases (OND) as controls. The samples collected from patients were screened for the presence of JCV and BKV DNA using a sensitive nested PCR technique (n-PCR). Owing to the high level of homology between the JCV and BKV genomic sequences, the n-PCR is capable of detecting the DNA of both viruses [Yang and Wu, 1979; Frisque et al., 1984; Ferrante et al., 1995]. In addition, other elements considered in the study consisted of routine CSF testing, immunological parameters, neurological symptoms, and neuroimaging evidence.

TABLE I. Epidemiological Characteristics of the 64 AIDS Patients With PML and Control Patients

	Males (n = 51)	Females (n = 13)	Total (n = 64)
Mean age in years (range)	31 (24–50)	30 (22–49)	31 (22–50)
Risk factors:			
IVDU	34 (67%)	7 (54%)	41 (64%)
Homosexual	5 (10%)	0	5 (8%)
Heterosexual with HIV-infected partner	1 (2%)	4 (31%)	5 (6%)
Transfusion-recipient	2 (4%)	0	2 (3%)
Not specified	9 (17%)	2 (15%)	11 (17%)

It should be noted that investigations concerned with AIDS patients with and without PML are particularly useful, as the rate of JCV and BKV reactivation can be established for the two study groups, both of which are clearly immunosuppressed, but differ in the type of neurological disease that has developed.

MATERIALS AND METHODS

Patients

Sixty-four AIDS patients suffering from various neurological diseases including PML, were enrolled in the study upon admission to the Departments of Infectious Diseases of the Hospitals of Pavia, Monza, and Busto Arsizio, all of which are located in northern Italy. The study population was made up of 51 males and 13 women. There were 12 PML cases, of whom 11 males and one female. The age range for was from 28 to 46 years for the PML group and 22 to 50 years for the control group. Some of the characteristics of these 64 AIDS patients are illustrated in Table I, including data on ages, gender, and risk factors. HIV infection onset ranged from less than 1 year to 10 years before the collection of the samples. As mentioned above, 12 patients presented clinical manifestations and neuroradiological findings indicating PML. Moreover, the PML diagnosis was confirmed at autopsy by histological examination. The other patients had been diagnosed with cerebral toxoplasmosis (11 patients), AIDS dementia complex (ADC) (four patients), cryptococcal meningitis (five patients), cerebral rhodococcosis (one patient), cytomegalovirus (CMV) encephalitis (seven patients), and encephalitis of unknown origin (six patients). The remaining 18 cases were patients with other neurological disorders, including paresis, cognitive, and visual deficits.

The risk factor in the 64 patients included 41 (64%) intravenous drug users (IVDUs), five (8%) homosexual males, five (8%) heterosexual partners of subjects at risk for HIV, and two patients (3%) that had HIV infection after blood transfusion. No risk factors were declared by 11 patients (17%). Among the PML pa-

tients, nine were IVDUs, two patients were homosexual males, and the woman was the partner of an HIV-positive individual.

All patients were classified according to the Centers for Disease Control and Prevention (CDC) system. The control patients were all classified in categories B or C, subcategories 1 or 2. The PML patients classified as follows: eight in subcategory C1, two in subcategory C2, and two in category B.

In addition to CSF examination, magnetic resonance imaging (MRI) and/or computed tomography were carried out on all patients.

CSF Laboratory Analysis

The CSF samples were obtained by lumbar puncture and immediately examined for cell count and to determine glucose level. The reference normal values adopted in our laboratory are ⩽4 cells/μl and 60–110 mg/dl, respectively. Part of each CSF sample was aliquoted immediately and stored at −80°C for testing later by n-PCR.

Blood-brain barrier (BBB) integrity, the IgG intrathecal synthesis indexes and the presence of IgG oligoclonal bands (OB) were evaluated using standard procedures. The Link and Tourtellotte IgG indexes and BBB integrity were calculated using an APS Beckman nephelometer (Beckman Instruments, Inc., Galway, Ireland) for the serum and CSF samples [Lefvert and Link, 1985; Tourtellotte et al., 1985]. Normal values were established as ⩽0.7 mg/ml/day for the Link index, ⩽3.3 mg/dl/day for the Tourtellotte index, and ⩽5.5 for BBB integrity.

The presence of IgG OB was evaluated by sodium dodecylsulfate (SDS) acrylamide gel electrophoresis after silver staining, using a PhastSystem apparatus (Pharmacia Biotech, Uppsala, Sweden).

Preparation of CSF, PBMCs, and Urine Samples

Samples of CSF, PBMCs, and urine were collected from each of the 64 patients, the samples being collected from the individual patient on the same day. Twenty μl of each CSF sample was added to a final volume of 50 μl of the n-PCR reaction mixture without any preliminary treatment. Aliquots of 2.5×10^6 cells of the PBMCs, separated by gradient centrifugation with Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) from heparinized blood, were suspended in 200 μl of lysis buffer containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 0.5% Tween 20, 0.5% NP40, 100 μg/ml proteinase K (Boehringer Mannheim, GmbH, Mannheim, Germany) and incubated at 56°C for 1 hr. Digestion was terminated by the inactivation of the enzyme at 95°C for 15 min. Twenty microliters of each lysate was added to the n-PCR reaction mixture.

Fresh urine samples were centrifuged for 10 min at 1,500 rpm and 5 μl of the urinary sediment was added to the n-PCR reaction mixture.

Amplification Protocol

A nested PCR was used for these experiments. The specificity and sensitivity of the n-PCR have been described in a previous study [Ferrante et al., 1995]. Primers for the outer n-PCR were JC1 and JC2 [Brouqui et al., 1992] and for the inner n-PCR PEP1 and PEP2 [Arthur et al., 1989].

PCR oligonucleotide primers were selected from within the JC virus genome encoding *large T antigen* and sharing significant genomic homology with BKV. The first round was carried out in a total volume of 50 μl containing 200 μM of each dNTP (Boehringer Mannheim GmbH, Mannheim, Germany), 1.5 mM $MgCl_2$, 10X reaction buffer (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Tween, 0.5% Nonidet P40), 2 U of *Taq* DNA polymerase (Perkin Elmer by Roche Molecular Systems Inc., Branchburg, NJ), 30 pmol of primers JC1 and JC2.

The outer amplification program provided for 30 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 1 min with a first initial denaturing step of 5 min. The inner PCR was carried out by adding 5 μl of the first amplification product to the PCR mixture containing 20 pmol of primers PEP1 and PEP2. The program included 30 cycles with annealing at 55°C.

All PCR reactions were carried out with one negative and one positive control. The negative control contained all the PCR components except the template. BKV DNA obtained from a brain tumor-derived cell line was used as the positive control. It was provided by the Institute of Histology and General Embryology of the University of Ferrara (Italy).

The amplified DNA was visualized by UV light exposure on a 3% agarose gel, after staining with ethidium bromide, yielding bands of 173 and 176 bp for JCV and BKV, respectively. In order to confirm the n-PCR results and to differentiate JCV from BKV DNA, all the positive PCR products were subjected to enzymatic restriction with *Bam* HI (Boehringer Mannheim GmbH, Mannheim, Germany), which cleaves the JCV amplified product into two fragments of 120 and 53 bp, whereas the BKV amplified product is visualized as only one fragment of 176 bp.

In addition, a colorimetric hybridization method (DEIA) was employed for the detection of the amplified DNA and this immunoassay was performed using biotinylated JCV- and BKV-specific probes (JEP and BEP) as described by Arthur et al. (1989). The experimental conditions, as well as the specificity and sensitivity of this method, have been defined and described in a previous study [Ferrante et al., 1995].

RESULTS

JCV DNA was detected by n-PCR in the CSF samples of 11 (92%) of the 12 AIDS patients with PML, whereas the other CSF samples from the remaining 52

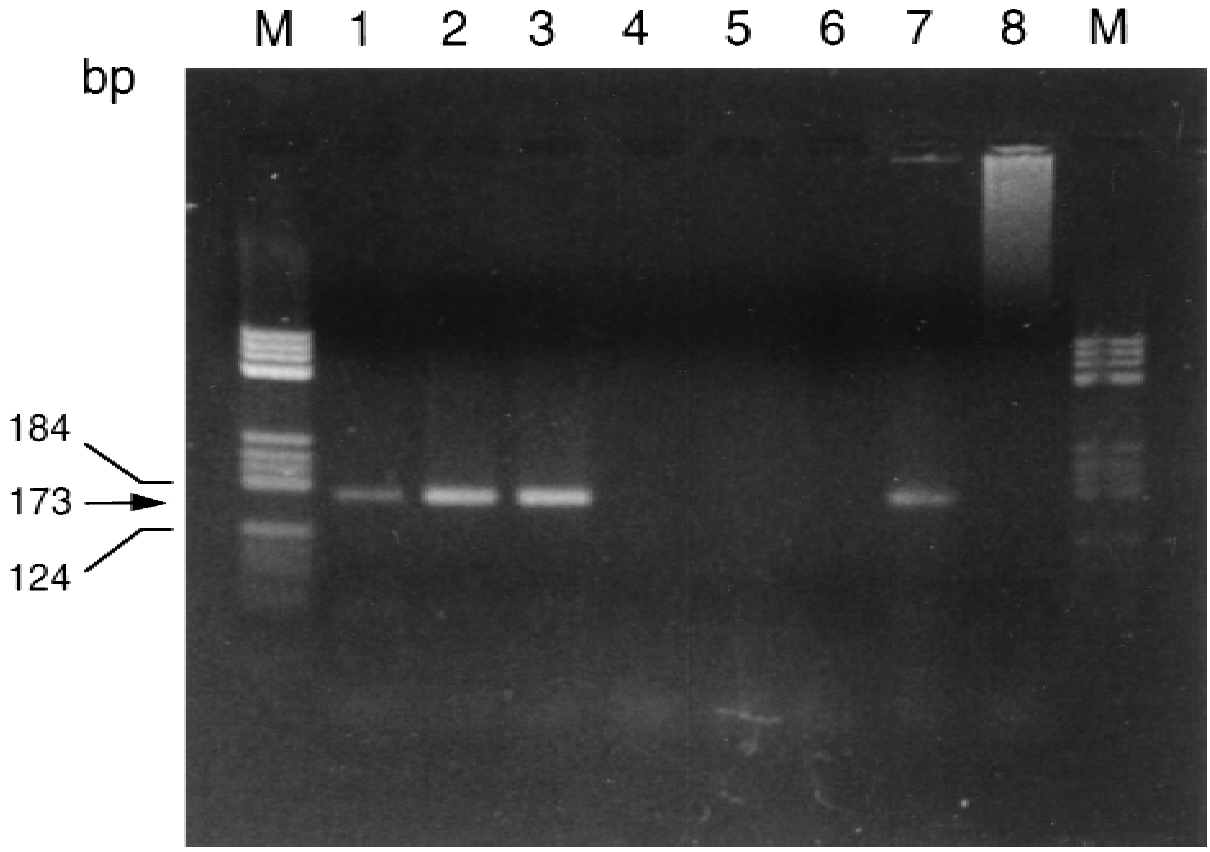

Fig. 1. Detection of amplified products analyzed on 3% agarose gel. **Lanes 1–3:** Urine, PBMCs, and CSF, respectively, from an AIDS patients with PML. **Lanes 4–6:** Urine, PBMCs, and CSF, respectively, of an AIDS patient without PML. **Lane 7:** Positive control. **Lane 8:** Negative control. M, DNA molecular weight marker (V, Boehringer Mannheim).

AIDS patients without PML were all negative. JCV DNA was found in the PBMCs from nine (75%) PML patients and from five (10%) of the other AIDS patients without PML, specifically in samples from two subjects with cryptococcal meningitis, two with paresis, and one with cognitive deficits. JCV viruria was observed frequently: JCV DNA was detected in the urine specimens of nine (75%) of the AIDS patients with PML and 15 (29%) of the AIDS patients without PML. Overall, the prevalence of JCV DNA in the study group of AIDS patients was equal to 11 (17%) of the CSF samples, 14 (22%) of the PBMC samples, and 24 (38%) of the urine specimens.

As the n-PCR employed in this study is capable of amplifying simultaneously JCV and BKV DNA, we were able to detect the presence of BKV DNA as well. The results showed the simultaneously presence of BKV DNA and JCV DNA in the urine specimens of three (6%) AIDS patients without PML, but who were affected by cerebral toxoplasmosis, cerebral rhodococcosis, and CMV encephalitis, respectively.

All the n-PCR products were analyzed by gel electrophoresis on 3% agarose and by DEIA. These methods yielded identical results, thereby confirming the high sensitivity of the n-PCR. Figure 1 contains the results of agarose gel electrophoresis on the CSF, PBMCs and urine samples from one PML patient who was JCV-DNA positive, and from one AIDS patient without PML, who was JCV-DNA negative.

The main epidemiological characteristics of the 12 PML cases are listed in Table II, along with the results of screening for JCV DNA in their CSF, PBMCs, and urine samples. It is possible to observe from the results of the investigation on the presence of JCV DNA by n-PCR, that the PML patient whose CSF samples was negative, had JCV DNA in both the PBMC sample and the urine specimen. In addition, of the three PML cases whose PBMC samples were negative, two had JCV DNA in both the CSF and urine samples. Two of the three patients with PML in whose urine specimens JCV DNA was not recovered, had the virus in both the CSF and PBMC samples. Only in the case of one PML patient was JCV DNA detected in the CSF, but not in the PBMC and urine specimens.

The clinical signs for the 12 AIDS patients with PML are reported in Table III, along with neurological findings and CSF laboratory data. The neurological symptoms were those recorded at the time of collection of the samples for screening the patients for JCV DNA. Among the PML patients, initial neurological manifestations included mono- or hemiparesis as the only symptom, or in association with other symptoms, as was the case for six subjects (50%). Cognitive deficits were observed in three cases (25%), whereas two sub-

TABLE II. Epidemiological Characteristics of the 12 PML Patients and JCV DNA Detection in Their CSF, PBMCs, and Urine Samples

Patient	Gender	Age	Years since HIV-infection	CDC category	Risk factor	JCV DNA detection		
						CSF	PBMC	Urine
1	M	30	9	IV C1	IVDU	+	+	+
2	M	29	6	IV C1	IVDU	+	+	+
3	M	28	<1	IV C1	homosexual	+	+	−
4	M	37	8	IV B	IVDU	+	+	+
5	M	39	10	IV C1	IVDU	−	+	+
6	M	34	<1	IV C1	IVDU	+	+	−
7	F	31	2	IV C1	risk partner	+	+	+
8	M	46	<1	IV C2	homosexual	+	−	+
9	M	29	<1	IV C1	IVDU	+	+	+
10	M	32	5	IV B	IVDU	+	+	+
11	M	33	10	IV C1	IVDU	+	−	+
12	M	30	5	IV C2	IVDU	+	−	−

TABLE III. Neurological Manifestations, Neuroradiological Findings, and CSF Laboratory Data for the Twelve PML Cases*

Patient	Neurological manifestations	Localization of lesions by MRI	CSF parameters					
			Glycorrachia	Cell count	BBB integrity	Link index	Tourtellotte index	Oligoclonal bands
1	cognitive deficits; monoparesis; seizures	parietal	52	0	7.2	0.58	2.27	—
2	cognitive deficits; speech deficits; headache	frontal, parieto-temporal	52	2.2	4.3	0.47	0	—
3	coma; seizures	cerebellum and frontal	49	1.2	7.4	0.49	0	—
4	hemiparesis; coma	parietal-occipital	46	2.6	5.4	0.47	0	—
5	cognitive deficit; headache	basal nuclei	47	0	7.7	0.73	16.8	n.p.
6	hemiparesis	cerebellum	56	1	10	0.59	7.92	+
7	hemiparesis	parietal-occipital	42	0	4.6	0.60	0	—
8	limb incoordination	parietal and frontal	56	0.8	3.8	0.54	0	—
9	hemiparesis; headache	corpus callosum, frontal and parietal	44	0.4	3.1	0.69	0	—
10	visual deficit; limb incoordination	cerebellum	50	0.4	11.2	0.57	6.55	—
11	limb incoordination	cerebellum	54	0	13.3	0.47	0	+
12	visual deficits; hemiparesis	cerebellum	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.

*n.p. = not performed; BBB = blood brain barrier.

jects had a speech deficit (one patient, 8%) and visual deficit (one patient, 8%) as their initial symptom, respectively. Headaches were recorded for three cases (25%) and coma, seizures, and limb incoordination were observed in two cases (17%).

As for the neuroradiological results obtained through MRI and CT, hypodense lesions were found in the parietal regions, associated with frontal, occipital, or temporal localization, in six (50%) of the PML patients. Involvement of the cerebellum was observed in five (42%) patients and demyelinating lesions were found in the basal nuclei of one (8%) PML patient.

CSF cell counts and glucose levels were normal in all the PML patients. An alteration of BBB integrity was observed in 50% of these cases. The Tourtellotte index proved to be above normal values in 25% of the cases, as did the Link index in the case of one patient (8%). Positive OB detection results were observed only in two cases (17%). Only one PML patient showed simultaneously higher BBB integrity and Link and Tourtel-

TABLE IV. Comparison of Some Epidemiological, Clinical, and Immunological Characteristics of the 64 AIDS Patients With PML and Control Patients

	N	Males (prevalence)	Mean age in years (range)	Duration of HIV-infection in years mean (range)	$CD4^+$ cell/ml mean (range)	Survival in months mean (range)
PML	12	11 (92%)	33 (28–46)	4 (0–10)	89 (0–253)	2.5 (<1–6)[a]
OND	52	40 (77%)	32 (22–50)	5 (0–11)	87 (0–720)	6 (<1–18)[a]
Total	64	51 (80%)	33 (22–50)	5 (0–11)	88 (0–720)	5.6 (<1–18)

[a]PML vs. OND: $P < .01$. The only statistically significant difference emerging from the comparison.

lotte indexes. However, the OB were not checked for this patient, due to the low amount of CSF collected.

The results obtained on the CSF parameters on the other 52 AIDS patients and controls are not shown here analytically. However, it should be noted that in the non-PML group, there was an alteration of BBB integrity in 75% of the cases, higher Tourtellotte and Link index values in 52% and 32% of the cases, respectively, and IgG OB were present in 43% of the CSF samples.

In an attempt to verify the possible influence of recognizable clinical and epidemiological aspects that could influence the course of JCV infection, a comparison was made between the 12 AIDS patients with PML and the 52 AIDS patients and controls (Table IV). The two groups did not differ significantly in age, nor in the duration of HIV infection. The level of total $CD4^+$ cells among the PML patients, with a mean value of 89 cells/ml was comparable to the mean of 87 cells/ml observed among the control patients. Statistically significant difference ($P < .01$) was observed between the two groups for survival time after collection of samples. In fact, the PML group's mean survival time was 2.5 months, whereas the control group's survival time was 6 months.

DISCUSSION

As a consequence of the increase in the incidence of PML due to the AIDS epidemic, more and more investigations are being conducted in an attempt to establish a correct and non-invasive approach serving for in vivo diagnosis of PML and to better understand its pathogenetic mechanism. This study saw the participation of a significantly large number of AIDS patients with concurrent neurological disorders and it was conducted with the objective of screening JCV DNA in CSF, PBMCs, and urine specimens collected simultaneously from individual patients at the onset of neurological symptoms. In addition, the study also included careful investigation of clinical, immunological, laboratory parameters, and neuroradiological imaging for all the subjects. Using a n-PCR, followed by agarose gel electrophoresis and non-isotopic DNA hybridization, which ensure the best results in terms of sensitivity and specificity [Ferrante et al., 1995], JCV DNA was found in 92% of the CSF samples from the PML patients, while no amplified products were obtained from the CSF samples collected from the 52 AIDS patients and controls.

Therefore, the method employed showed a sensitivity of 92% for the diagnosis of PML, a percentage that is comparable to that obtained by some authors [Moret et al., 1993; Bogdanovic et al., 1994], but also higher than that observed by others [Weber et al., 1994]. The 100% specificity observed in this study is in keeping with previous observations [Gibson et al., 1993; Bogdanovic et al., 1994; Weber et al., 1994; Cinque et al., 1996]. However, it is also higher than that reported in other studies [Gibson et al., 1993; Fong et al., 1995]. On the whole, the results obtained by n-PCR for the detection of JCV DNA in CSF samples clearly indicate its usefulness for *in vivo* diagnosis of PML.

The data resulting from the PBMCs analysis are also of particular interest. JCV DNA was found in 75% of the samples from AIDS patients with PML and only in 10% of the samples from the non-PML subjects. The incidence of JCV DNA observed in the PBMC samples from patients with and without PML is lower than that reported (89.5% and 38%, respectively) in a recent study [Tornatore et al., 1992]. It should be noted that while some authors report failure to detect JCV DNA in the PBMCs of HIV-infected subjects [Sundsfjord et al., 1994], others have achieved the outstanding rates of 78% in the PBMCs of healthy immunocompetent adults and of 80% in immunocompetent patients with central nervous system diseases unrelated to PML [Dorries et al., 1994].

Several studies have been undertaken to analyze the incidence of JCV viruria in immunodepressed and immunocompetent subjects [Yogo et al., 1990; Flaegstad et al., 1991; Arthur et al., 1989; Markowitz et al., 1993; Kitamura et al., 1990]. In our study, the frequency of JCV viruria was significantly higher among the PML patients (75%), compared to the AIDS patients with OND (29%). The incidence of JCV viruria among the AIDS patients without PML in our study is comparable not only to that observed among HIV-positive immunodepressed patients [Markowitz et al., 1991; Sundsfjord et al., 1994], but also to that observed previously among immunocompetent individuals [Bogdanovic et al., 1994; Kitamura et al., 1990; Markowitz et al., 1991; Sundsfjord et al., 1994]. The presence of BKV viruria in 6% of the AIDS group without PML, is also in keeping with previous reports, which have provided evidence of the frequent presence of BKV in the urine of immunosuppressed and healthy subjects [Markowitz et al., 1991, 1993].

Some epidemiological, clinical, and immunological parameters were also taken into account to weigh their possible impact on the presence or absence of JCV in these two groups of patients. No differences between the two groups were observed as regards gender, age, duration of HIV-infection or $CD4^{+}$ cell count. This suggests that besides immunodepression, other factors probably related to the biological characteristics of JCV, are required for the development of PML.

As noted above, the only significant difference between the two groups was the survival period: it proved to be shorter for the PML patients, compared to the OND group. In fact, the mean survival time for the PML group was 2.8 months, with a maximum of 6 months, compared to the 6 months, with a maximum of 18 months, for the controls. These findings confirm those reported by other researchers [Brooks and Walker, 1984; Kuchelmeister et al., 1993; Von Einsiedel et al., 1993; White et al., 1992], while also confirming that PML is usually a rapidly developing, progressive, fatal and inexorable disease.

The neurological symptoms observed among the PML patients, with a high prevalence of paresis and a lower prevalence of cognitive deficits, headache, coma, seizures and limb incoordination, do not differ from those described by other authors [Berger et al., 1987; Brooks and Walker, 1984; Von Einsiedel et al., 1993]. The same may be said for the neuroradiological evidence resulting from the CT and MRI, with the results being similar to previous reports [Whiteman et al., 1993]. The only difference was a high incidence of demyelinating lesions in the frontal area and also in the cerebellum, among the PML subjects.

Again, as in previous reports [Berger et al., 1987; Brooks and Walker, 1984; Von Einsiedel et al., 1993], a typical PML-related pattern did not emerge from the routine tests performed on CSF samples in this study. Alterations of CSF glucose levels and cell counts were not observed among the PML cases. On the contrary, the incidence of the presence of IgG OB and abnormal values on the intrathecal IgG synthesis indexes and for BBB integrity, were significantly more frequent in the group of AIDS patients without PML, but affected by OND.

One aspect of the pathogenesis of PML that still requires clarification, regards the times and modalities according to which the virus reaches the CNS and induces PML. The observation reported by our team and by others [Ferrante et al., 1995; White et al., 1992; Vago et al., 1996] concerning the presence of JCV DNA in the brains of PML patients, but also of subjects whose deaths were attributable to other causes, supports the legitimate suspicion that as a consequence of immunosuppression, PML may arise from the reactivation of JCV already present in the brain, perhaps even at the onset of the primary infection.

Given that in our study, no differences were revealed between the AIDS patients with and without PML in terms of the $CD4^{+}$ count, if the hypothesis of reactivation of JCV already present in the brain were indeed true, we could expect to find a greater frequency of JCV DNA in the CSF of PML patients, compared to the control patients and according to this logic, no differences in the frequency of JCV DNA detected in other body areas. Instead, the data obtained demonstrate that JCV DNA is present and with a significantly higher frequency in patients with PML, compared to the control patients, not only in the CSF, but also in the PBMCs and urine specimens. In our opinion, these findings support the hypothesis that in AIDS patients, PML develops as a consequence of a diffuse reactivation of the JCV infection and that JCV could be brought into the CNS by means of infected blood cells.

In conclusion we may state that the results of this study confirm the development of PML as a specific AIDS-related problem and show that the n-PCR is a useful and reliable tool for the diagnosis of PML. An extensive use of this approach and molecular characterization of amplified JCV DNA may very well be passkeys for our future understanding of the pathogenetic mechanisms of PML.

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